Kidney International, Vol. 51 (1997), pp. 1088–1099

Mesangial cell proliferation mediated by PDGF and bFGF is determined by levels of the cyclin kinase inhibitor $p27^{Kip1}$

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Mesangial cell proliferation mediated by PDGF and bFGF is determined by levels of the cyclin kinase inhibitor $p27^{Kip1}$. Mesangial cell proliferation *in vitro* is regulated by many cytokines. Platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) are potent mesangial cell mitogens, whereas transforming growth factor-β1 (TGF-β1) reduces their effects. We examined how these cytokines regulate rat mesangial cell proliferation at the level of the cell-cycle. Quiescent mesangial cells *in vitro* express the cyclin kinase inhibitor, $p27^{Kip1}$ (p27), and PDGF- and bFGF-induced mesangial cell proliferation is associated with a substantial decrease in p27 levels. Consequently there is a marked increase in expression (Western blot analysis, immunostaining) of cyclin A and CDK2. The decline in p27 levels was prevented by TGF-β1 during inhibition of PDGF- and bFGF-induced mesangial cell proliferation. To determine the functional role of p27 during cytokine-mediated mesangial cell proliferation, the expression of p27 was reduced with specific $p27^{Kip1}$ antisense oligodeoxynucleotides. Reducing the levels of p27 resulted in an increased magnitude of mesangial cell proliferation (BrdU and ^{3}H-thymidine incorporation) induced by PDGF and bFGF compared to non-transfected mesangial cells and mesangial cells transfected with control mismatch oligodeoxynucleotides. Furthermore, the onset of maximal proliferation occurred earlier in mesangial cells transfected with antisense compared to control. The reduction in proliferation by TGF-β1 were not altered by decreased p27 expression. Reducing p27 expression in the absence of mitogens was not associated with entry into the cell-cycle. These results suggest cytokine mediated mesangial cell proliferation is associated with specific cell-cycle proteins, and that the levels of p27 may be important in determining the mesangial cell's proliferative response to PDGF and bFGF *in vitro*.

Mesangial cell (MC) proliferation characterizes many forms of glomerulonephritis [1]. MC proliferation can be induced by many mitogenic cytokines, including platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) [2, 3]. Abboud and colleagues were the first to show that MC *in vitro* express PDGF, and that PDGF AB and BB dimers induce MC proliferation *in vitro* [4]. Silver reported that growth factors such as EGF, TGF-α, bFGF and PDGF itself induce the expression of PDGF A and B chain [5]. Furthermore, blocking PDGF with anti-PDGF antibody abrogated the mitogenic effect of EGF-induced MC proliferation, suggesting that PDGF is a critical autocrine regulator of MC proliferation *in vitro*. bFGF is also produced by MC, and is a potent MC mitogen *in vitro* [6] and *in vivo* [7]. Moreover, PDGF and bFGF increase MC proliferation *in vivo* in experimental mesangial proliferative glomerulonephritis [7]. PDGF and bFGF both signal through a tyrosine kinase receptor [8], while some of the known nuclear events include activation of the transcription factors c-*myc* and c-*fos* [9].

Transforming growth factor-β1 (TGF-β1) is a potent inhibitor of proliferation in a variety of cell types, including MC [10, 11]. Furthermore, TGF-β1 reduces MC proliferation induced by PDGF and bFGF [12]. Although recent studies have delineated the binding of TGF-β1 to different serine-threonine receptor subtypes [reviewed in 13], little is known about the signaling pathways mediating TGF-β1 [14].

Cell proliferation is ultimately governed at the level of the cell-cycle. Under appropriate stimuli, cells exit quiescence (G0 phase) and enter the cell-cycle at G1 [15]. Progression through the cell-cycle is dependent on the balance of positive and negative regulatory cell-cycle proteins [16, 17]. During each phase of the cell-cycle there is an increase in expression for specific cyclins, which bind to their catalytic partners, the cyclin-dependent kinases (CDK) [reviewed 18]. This results in the formation of active cyclin-CDK complexes, which are responsible for the phosphorylation of the retinoblastoma protein as well as other substrates, an event required for G1/S transition and DNA synthesis [19]. The D-cyclins bind CDK4 in G1, whereas cyclin E and CDK2 complex at G1/S transition [20]. Cyclin A is complexed with CDK2 during the S phase [21].

Cyclin-kinase inhibitors (CKI) negatively regulate the cell-cycle by inhibiting cyclin-CDK complexes [reviewed in 22]. One family of CKI include $p21^{Cip1}$ and $p27^{Kip1}$ (abbreviated as p27 in this manuscript), and the N-termini of these CKI share homology, and can bind to and inhibit CDK. Overexpression of these CKI causes cell-cycle arrest in some non-renal cells [23]. In certain cell types, p27 levels oscillate throughout the cell cycle, while in others there is no change during proliferation [24].

Although the expression of cell-cycle proteins has been studied in some non-renal cells, the expression of cell cycle regulatory proteins in glomerular cells is poorly understood, and it is unclear if all mitogens are associated with the same cell cycle events. Accordingly, the first aim of the current study was to determine the expression of cell cycle regulatory proteins in the glomerular MC *in vitro*, while a second aim was to determine the expression

Received for publication October 8, 1996
and in revised form November 22, 1996
Accepted for publication November 25, 1996

of specific cell-cycle proteins following stimulation by the mitogenic cytokines, PDGF and bFGF. Our results show that PDGF and bFGF induced MC proliferation is associated with increased CDK2 expression, which is not typical of non-renal cells. We show that MC proliferation is associated with a decrease in p27 protein levels, but unlike other cell types, the levels were always detectable. This result formed the basis for the third aim of the current study, in which we determined whether the levels of p27 determine the MC proliferative response to mitogenic and anti-proliferative cytokines. Reducing the expression of p27 in MC with specific oligodeoxynucleotides (ODN) to p27 enhanced the magnitude of PDGF and bFGF induced MC proliferation. Finally, we sought to determine which cell-cycle proteins are altered during TGF-β1 inhibition of MC proliferation. Although TGF-β1 prevents the decrease in p27 levels induced by PDGF and bFGF in MC, reducing p27 with antisense ODN did not alter the anti-proliferative action of TGF-β1. These results suggest that MC proliferation is controlled by a complex interplay of positive and negative cell-cycle regulatory proteins. p27 is a critical determinant in the MC response to mitogens, but by itself is not responsible for the anti-proliferative effects of TGF-β1.

Methods

Establishment of rat mesangial cells

Rat mesangial cells (MC) were grown in culture as previously described [25]. In brief, glomeruli were isolated by differential sieving from kidneys of six male Sprague-Dawley rats (50 to 100 grams), and grown in RPMI media (Irvine Scientific, Santa Ana, CA, USA) that contained 15% fetal calf serum (Summit Biotechnology, Ft. Collins, CO, USA), 15 mM HEPES (Sigma Chemical Co., St. Louis, MO, USA), 89 μg/ml sodium-pyruvate (Irvine Scientific), 200 μM L-glutamine (Irvine Scientific), 81 μg/ml penicillin G, 81 μg/ml streptomycin sulphate (Irvine Scientific) and 0.66 U/ml insulin (Gibco BRL, Grand Island, NY, USA). MC were passaged every 72 to 96 hours by trypsinization. MC were identified by their typical stelate morphology, and by immunostaining for characteristic cytoskeletal filament proteins (desmin and α-actin), cell-surface antigens (Thy1, thrombospondin) and negative staining with antibodies to the leukocyte common antigen OX-1 or factor VIII [26]. We used passages 10 to 20 MC, and each experiment was performed a minimum of six times.

Experimental design

Inducing mesangial cell proliferation. To synchronize MC in a quiescent state, MC were plated at a density of 3×10^5 MC/cm^2 in serum free media for 48 hours. To study protein expression, MC were grown in 10 cm^2 plates, while 24-well plates were used for the proliferation assay using ^{3}H-thymidine or 5-bromo-2′-deoxyuridine incorporation into DNA. Pilot studies using flow cytometry determined that these conditions resulted in $\geq$ 90% of MC being in the G0/G1 phase. To study the temporal expression of cell cycle regulatory proteins following mitogen stimulation of quiescent MC, the media was replaced at 48 hours with serum free media containing PDGF BB (PDGF) (concentration of 10 ng/ml; Becton Dickinson Labware, Bedford, MA, USA), or bFGF (concentration of 10 ng/ml; Becton Dickinson Labware), and studies were performed at 0, 6, 12, 18 and 24 hours following stimulation. To determine the expression of cell-cycle proteins following growth inhibition by TGF-β1, separate experiments were performed in which TGF-β1 (concentration 1 ng/ml; R&D Systems, Minneapolis, MN, USA) was added to the media containing PDGF (10 ng/ml) or bFGF (10 ng/ml), and studies were performed at 0, 12, 18 and 24 hours following stimulation.

Assessment of MC proliferation

The incorporation of ^{3}H-thymidine and 5-bromo-2′-deoxyuridine (BrdU) into DNA was used as a measure of MC proliferation. A pulse of 2 μCi of ^{3}H-thymidine (Dupont NEN Research Products, Boston, MA, USA) was added to each well for the final four hours of cytokine stimulation at each time point. MC were washed three times with cold PBS then incubated in ice-cold 10% trichloroacetic acid for 25 minutes, followed by ether-alcohol for five minutes. The cells were solubilized with 0.2 M NaOH, neutralized with 0.4 M glacial acetic acid and ^{3}H-thymidine was counted on a Beckman LS 7500 scintillation counter. To ensure that the ^{3}H-thymidine incorporation was standardized for cell number, cell protein concentrations were determined in each well using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. ^{3}H-thymidine incorporation is reported as counts per minute/μg protein.

To determine the number of MC undergoing entry into S phase at 24 hours, we labeled MC with BrdU (Cell proliferation kit; Amersham Life Science, Arlington, IL, USA) for three hours (1:500 dilution), and performed immunostaining according to the manufacturer's directions. Detection of bound antibody was achieved using a peroxidase-conjugated monoclonal antibody, followed by Diaminobenzidine with cobalt and nickel. Camco quick stain II (Baxter Scientific Products, McGaw Park, IL, USA) was used as a counterstain. To determine the percentage of cells staining positive for BrdU, 200 MC were counted in a blinded fashion at each time point, and the percentage of MC staining positive for BrdU was determined.

Determining the expression of cell-cycle regulatory proteins in mesangial cells

The levels of cell-cycle regulatory proteins were determined by immunostaining and Western blot analysis.

Immunostaining. MC were grown on four-well chamber slides, and fixed in 3.7% paraformaldehyde for 10 minutes at room temperature, then washed three times in cold PBS. The cells were permeablized with 0.5% NP-40 buffer for 10 minutes at room temperature. Following a wash in PBS, the following primary rabbit polyclonal antibodies were incubated overnight at 4°C: cyclin A (dilution 1:1000; provided by J.M.R.), CDK2 (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p27 (dilution 1:1000; provided by J.M.R.). Controls included omitting the primary antibody, replacing the primary antibody with an irrelevant antibody of the same species and peptide absorption (the source for the peptides was the same as the primary antibody). A secondary biotinylated goat anti-rabbit antibody was added (1:400 dilution, Vector Laboratories, Burlingame, CA, USA) for 60 minutes at room temperature, followed by a FITC-conjugated streptavidin (1:32 dilution; Cappel Research Products, Durham, NC, USA) for 60 minutes at room temperature. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (Boehringer Mannheim, Indianapolis, IN, USA). The number of FITC-positive cells were expressed as a percentage of the total number of cells.

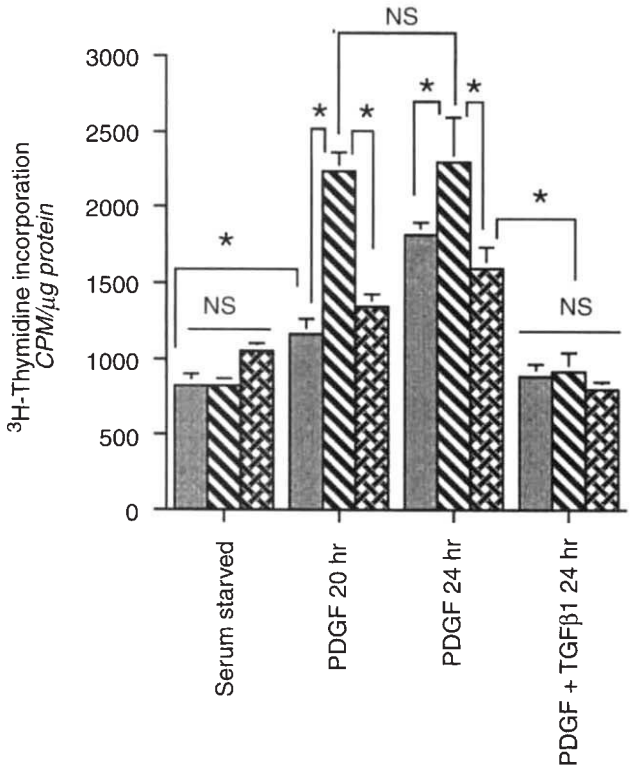

Fig. 1. *Effect of PDGF on rat mesangial cell (MC) proliferation using ^{3}H-thymidine proliferation assay.* MC proliferation was determined by ^{3}H-thymidine incorporation into DNA corrected for protein concentration in untransfected MC (▨), MC transfected with antisense oligodeoxynucleotide (ODN) to $p27^{Kip1}$ (p27) which lowered p27 levels (▧), and in MC transfected with mismatch ODN to p27, used as a control for the antisense ODN (▩). Serum-starved or quiescent MC were stimulated with PDGF alone (10 ng/ml), or with PDGF (10 ng/ml) plus TGF-β1 (1 ng/ml). In non-transfected MC, PDGF increased MC proliferation at 20 hours (20 hr), and proliferation was maximal at 24 hours (24 hr), whereas TGF-β1 reduced PDGF induced MC proliferation at 20 hours and 24 hours. There was no significant difference (NS) in ^{3}H-thymidine incorporation among the three groups when serum-starved. Antisense to p27 was associated with a significantly increased MC proliferation at 20 hours and 24 hours following PDGF stimulation compared to untransfected MC, and MC transfected with mismatch ODN. The onset of maximal MC proliferation was also earlier (20 hr) following antisense transfection. TGF-β1 reduced proliferation in transfected and non-transfected MC. $^*P < 0.05$.

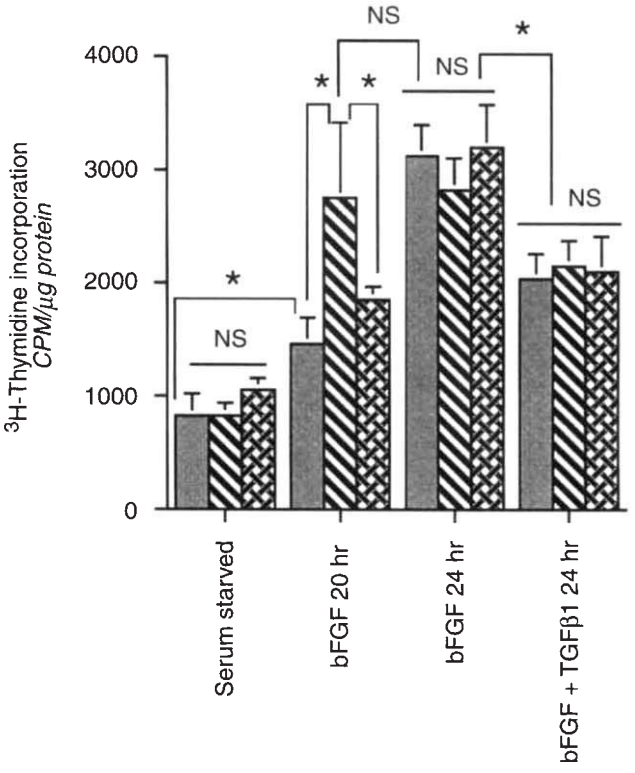

Fig. 2. *Effect of bFGF on rat mesangial cell (MC) proliferation using ^{3}H-thymidine proliferation assay.* MC proliferation was determined by ^{3}H-thymidine incorporation into DNA corrected for protein concentration, in untransfected MC (▨), MC transfected with antisense oligodeoxynucleotide (ODN) to $p27^{Kip1}$ (p27) that lowered p27 levels (▧), and in MC transfected with mismatch ODN to p27, used as a control for the antisense ODN (▩). Serum-starved or quiescent MC were stimulated with bFGF alone (10 ng/ml), or with bFGF (10 ng/ml) plus TGF-β1 (1 ng/ml). In non-transfected MC, bFGF increased MC proliferation at 20 hours (20 hr), and proliferation was maximal at 24 hours (24 hr), whereas TGF-β1 reduced, but did not prevent bFGF induced MC proliferation at 20 hours and 24 hours. There was no significant difference (NS) in ^{3}H-Thymidine incorporation among the three groups when serum-starved. Antisense to p27 was associated with a significantly increased MC proliferation at 20 hours following bFGF stimulation compared to untransfected MC, and MC transfected with mismatch ODN. At 24 hours there was no difference in MC proliferation in MC transfected with antisene or mismatch. TGF-β1 reduced proliferation in transfected and non-transfected MC. $^*P < 0.001$.

Western blot analysis. MC were washed three times with cold PBS, trypsinized and following centrifugation, the pellet was resuspended in a buffer containing 1% triton, 10% Glycerol, 20 mM HEPES, 100 mM NaCl with 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, 0.1 mM sodium orthovanadate and 50 mM sodium fluoride (reagents purchased from Sigma Biosciences, St. Louis, MO, USA). Following a vortex, the MC were placed on ice for 15 minutes. The cells were centrifugated at 14,000 rpm for five minutes, and the protein concentration of the supernatant was measured by BCA protein assay (Pierce, Rockford, IL, USA). For Western blot analysis, 20 μg of MC protein extract was loaded in each lane, separated under reducing conditions on a 15% SDS-PAGE gel, and transferred to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting [6]. To reduce nonspecific antibody binding, the membranes were first blocked with 5% non-fat dried milk for 30 minutes at room temperature. This was followed by a one hour incubation at room temperature with antibodies to either cyclin A, CDK2 or p27. Controls included omitting the primary antibody, replacing the primary antibody with rabbit serum and peptide absorption. An alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI, USA) was used for detection with the chromagen, 5-Bromo-4-Chloro-3-Indolyl phosphate/nitro blue tetrazolium (Sigma Biosciences). The gels were stained with Coomassie blue to check for complete protein transfer, and following Western blot analysis, filters were also stained with Coomassie blue to ensure that protein loading was equal.

Immunoprecipitation studies. To determine if p27 associated with cyclin A-CDK2 complexes during PDGF induced MC proliferation, we performed immunoprecipitation [29]. MC protein

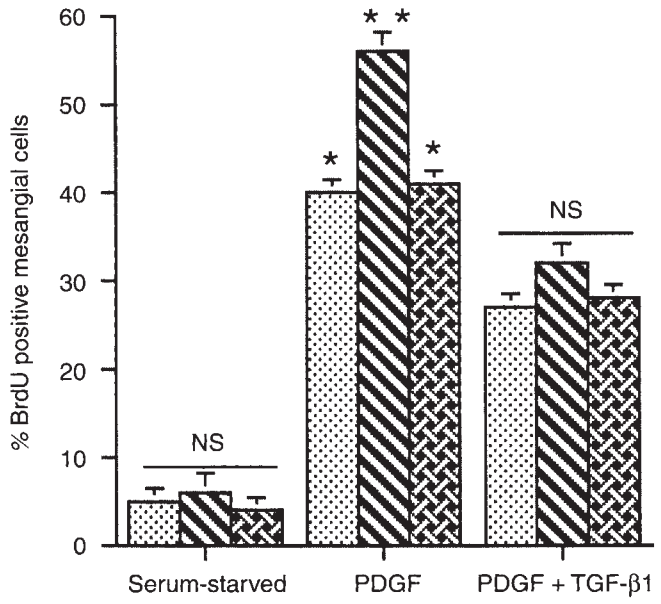

Fig. 3. *Mesangial cell (MC) entry into S phase following stimulation by PDGF.* In MC transfected with p27 antisense oligodeoxynucleotides or with control mismatch oligodeoxynucleotides, and in non-transfected MC, entry into the S phase of the cell-cycle was determined by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation into DNA. There was no difference (NS) in BrdU staining in non-transfected MC and transfected MC under serum-starved conditions, where less than 10% of cells stained positive for BrdU. At 24 hours of PDGF (10 ng/ml) stimulation, there was increased BrdU staining in non-transfected and transfected MC compared to serum-starved conditions ($*P < 0.05$). In MC transfected with antisense the magnitude of proliferation was increased compared to MC transfected with mismatch or non-transfected MC ($**P < 0.05$). TGF-β1 (1 ng/ml) reduced but did not prevent PDGF induced MC proliferation in all three groups. Symbols are: (▨) non-transfected; (▧) oligodeoxynucleotide; (▩) mismatch.

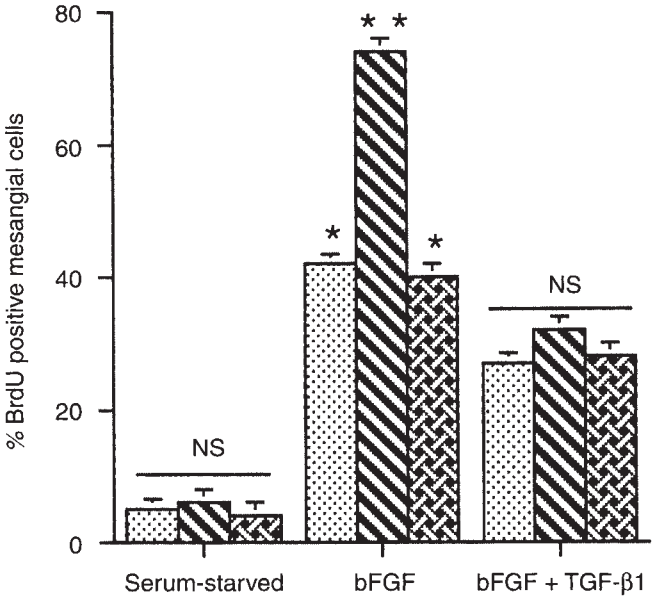

Fig. 4. *Mesangial cell (MC) entry into S phase following stimulation by bFGF.* In MC transfected with p27 antisense oligodeoxynucleotides or with control mismatch oligodeoxynucleotides, and in non-transfected MC, entry into the S phase of the cell-cycle was determined by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation into DNA. At 24 hours of bFGF (10 ng/ml) stimulation, there was increased BrdU staining in non-transfected and transfected MC compared to serum-starved conditions ($*P < 0.05$). In MC transfected with antisense, the magnitude of proliferation was increased compared to MC transfected with mismatch or non-transfected MC ($**P < 0.05$). TGF-β1 (1 ng/ml) reduced but did not prevent PDGF induced MC proliferation in all three groups. Abbreviation NS is not significant. Symbols are: (▨) non-transfected; (▧) oligodeoxynucleotide; (▩) mismatch.

(200 μg) from each time point was incubated with the antibody to cyclin A (1:200 dilution) for 30 minutes at 4°C. Protein A sepharose beads (50 μl; Repligen, Cambridge, MA, USA) were added to each immunoprecipitation and incubated for 30 minutes at 4°C. Negative controls included substituting for the primary antibody with pre-immune rabbit serum. Reducing sample buffer was added to each tube, the samples were boiled for five minutes and the supernatant was then separated on a 15% SDS-PAGE and transferred to a PVDF membrane (Millipore), where it was incubated with an antibody to p27.

Reducing the levels of p27 with anti-sense

To determine the functional role of p27 in MC *in vitro* in response to proliferative and anti-proliferative cytokines, we undertook studies to reduce the expression of p27 in MC using antisense oligodeoxynucleotide transfection as recently described [27]. The oligodeoxynucleotides were synthesized on an automated synthesizer (model 8750; Milligen Bioresearch, Bedford, MA, USA), and the antisense sequence used was 5′-UGG CU-CUCC UGC GCC-3′, and the mismatch sequence was 5′-UCC CUU UGG CGC GCC-3′. We have shown that the mismatch oligodeoxynucleotides used in the current study are specific for p27, and do not effect the expression of other cell-cycle proteins [27]. The oligodeoxynucleotides were mixed with the liposome GS2888 cytofectin (Gilead Scientific, Foster City, CA, USA) as previously described [27].

Pilot studies were performed to optimize the conditions for the current study. To determine the concentration of ODN required to reduce the expression of p27, studies were undertaken with ODN at concentrations of 100, 50, 25, 10 and 1 nmol, each with a different concentration of liposome ranging from 1 to 5 μg/ml. Levels of p27 were measured by Western blot analysis. To assess the cytotoxicity of each concentration of ODN and liposome, the release of lactate dehydrogenase was measured using the LDH KIT (Sigma). To determine the efficiency of MC transfection by the ODN, we used FITC-labeled ODN and measured the percentage of MC nuclei that stained positive.

Based on these preliminary studies (see **Results** section), we used the following experimental design in the current study. MC were plated in 15% FCS at a density of 3×10^5 cells/cm^2 and allowed to adhere overnight. MC were then washed three times with serum free media, and the MC were left in serum free media for 18 hours. At this time, the media was replaced with serum free media containing either antisense ODN (1 nmol of antisense ODN complexed with 1 μg/ml liposome), mismatch (1 nmol of mismatch ODN complexed with 1 μg/ml liposome), or media alone for six hours. MC were then washed three times with serum free media, and the media was changed to serum free media containing either PDGF (10 ng/ml), bFGF (10 ng/ml), PDGF (10 ng/ml) and TGF-β1 (1 ng/ml), bFGF (10 ng/ml) and TGF-β1 (1

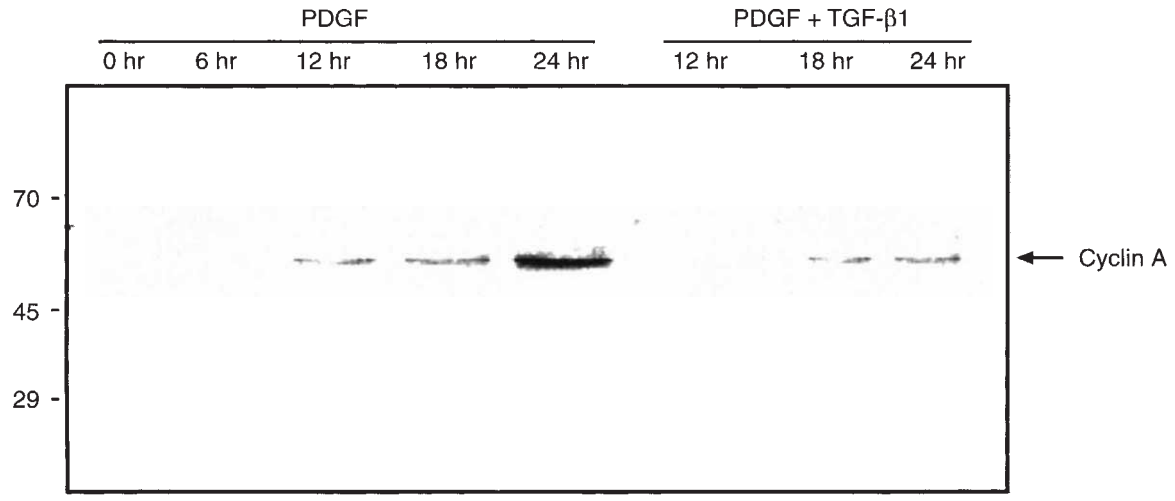

Fig. 5. *Effect of cyclin A expression measured by Western blot analysis in mesangial cells stimulated with PDGF.* Mesangial cells (MC) were serum-starved to achieve quiescence, then stimulated with either PDGF alone (10 ng/ml), or PDGF with TGF-β1 (1 ng/ml). Twenty micrograms of MC protein was loaded in each lane. Cyclin A protein was not detected in quiescent MC (lane 1). There was a progressive increase in cyclin A protein following stimulation with PDGF, at 12 hours (lane 3) and 18 hours (lane 4), which was maximal at 24 hours (lane 5), when MC proliferation is at a peak. In contrast, TGF-β1, which reduces PDGF induced MC proliferation, prevents the increase in cyclin A expression induced by PDGF at 12 hours (lane 5), and reduces the increase in cyclin A levels at 24 hours (lane 7). Protein molecular weight markers (in kDa) are shown in the left margin.

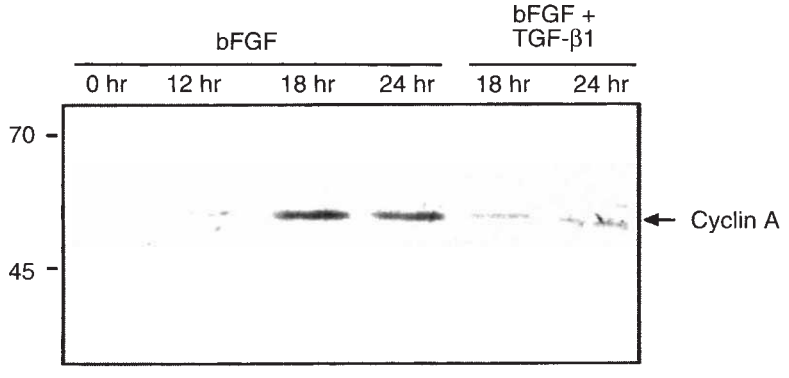

Fig. 6. *Effect of bFGF on cyclin A expression by Western blot analysis.* Mesangial cells (MC) were serum-starved to achieve quiescence, then stimulated with either bFGF alone (10 ng/ml), or bFGF with TGF-β1 (1 ng/ml). Twenty micrograms of MC protein were loaded in each lane. Cyclin A protein was undetected in quiescent MC (lane 1). There was a progressive increase in cyclin A protein following stimulation with bFGF, which was detected at 12 hours (lane 2), and maxiaml at 18 hours (lane 3) and 24 hours (lane 4), when MC proliferation is at a peak. In contrast, TGF-β1, which reduces bFGF induced MC proliferation, prevents the increase in cyclin A expression induced by bFGF at 18 hours (lane 5) and 24 hours (lane 6). Protein molecular weight markers (in kDa) are shown in the left margin.

ng/ml), or serum free media alone. Proliferation was assessed at 12, 16, 20 and 24 hours after cytokine stimulation by 3H-Thymidine and BrdU incorporation as described earlier. Each experiment was performed a minimum of six times.

Statistics

All values are expressed as mean ± SEM, unless otherwise stated. Statistical significance ($P < 0.05$) was evaluated using the Student's *t*-test or one way analysis of variance with modified *t*-test performed using the Bonferroni correction [28].

Results

MC proliferation studies in non-transfected MC

The effects of PDGF, bFGF and TGF-β1 stimulation on rat MC proliferation was assessed by measuring ^{3}H-thymidine and BrdU incorporation into DNA. Figures 1 and 2 show the results of ^{3}H-thymidine incorporation, and similar to studies by others [5, 7], PDGF ($P < 0.05$ vs. control) and bFGF ($P < 0.05$ vs. control) induce maximal DNA synthesis by 24 hours in non-transfected rat MC. The addition of TGF-β1 to PDGF or bFGF stimulated MC reduced MC proliferation ($P < 0.05$ vs. PDGF alone; $P < 0.05$ vs. bFGF alone), an observation that has been made previously [4–6, 12].

BrdU staining was used to determine the number of MC in S phase of the cell-cycle following stimulation by PDGF and bFGF with and without TGF-β1, and the results are shown in Figures 3 and 4. In serum-starved non-transfected MC, less than 10% of MC stained positive for BrdU. Following stimulation with PDGF 40 ± 1.5% of non-transfected MC stained positive for BrdU ($P < 0.05$ vs. control) and 42 ± 1.9% stained positive for BrdU following bFGF stimulation ($P < 0.05$ vs. control). Similar to the results of the ^{3}H-thymidine incorporation assay shown earlier, the addition of TGF-β1 to PDGF or bFGF stimulated MC reduced, but did not completely prevent entry into the S phase ($P < 0.05$ vs. PDGF alone; $P < 0.05$ vs. bFGF alone).

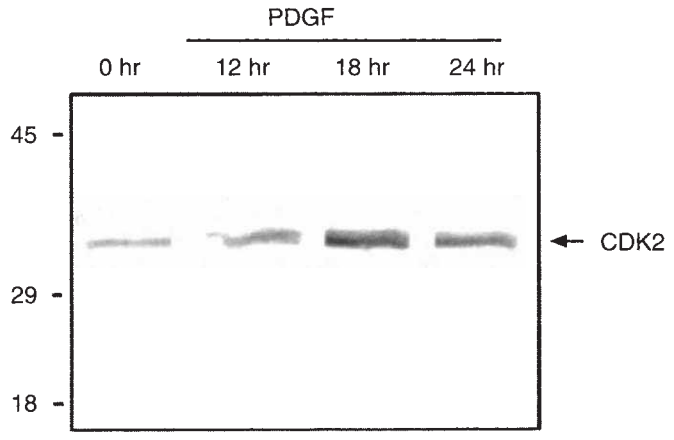

Fig. 7. *Western blot analysis for cyclin dependent kinase 2 (CDK2).* Mesangial cells (MC) were serum-starved to achieve quiescence, and then stimulated with PDGF (10 ng/ml). A Western blot analysis was performed on MC protein (20 μg in each lane) with an antibody to CDK2. Quiescent MC (lane 1) express low levels of CDK2. Following stimulation with PDGF, there is an increase in CDK2 at 12 hours (lane 2), 18 hours (lane 3) and 24 hours (lane 4). Protein molecular weight markers (in kDa) are shown in the left margin.

Cyclin A expression increases during mesangial cell proliferation

We determined the protein expression of cyclin A by Western blot analysis and immunostaining. Figure 5 shows a Western blot analysis for cyclin A protein in MC stimulated by PDGF. Cyclin A protein was undetectable in serum-starved MC, but there was a progressive and substantial increase in cyclin A expression during PDGF induced proliferation, first detected at 12 hours, and was maximal at 24 hours. No cyclin A staining was detected in quiescent MC. In contrast, the onset of MC proliferation was associated with an increase in the number of cells with positive intranuclear staining for cyclin A, first observed at 12 hours following stimulation, and was maximal at 24 hours. Following 24 hours of PDGF stimulation 30% of MC expressed cyclin A ($P < 0.05$ vs. serum-starved MC). A similar increase in cyclin A levels during MC proliferation following bFGF stimulation was seen by Western blot analysis (Fig. 6). Coommasie staining showed that the amount of protein loaded in each lane in all filters were equal.

CDK2 expression increases during mesangial cell proliferation

We determined the levels of CDK2 protein by Western blot analysis and immunostaining. There was a low level of protein expression for CDK2 in quiescent MC, where less than 10% of cells were stained with the CDK2 antibody (results not shown). PDGF and bFGF induced proliferation was associated with a progressive and marked increase in the number of MC staining for CDK2. At 12 hours 20% of cells were stained with the CDK2 antibody, while at 24 hours 30% of cells exhibited positive staining for CDK2 ($P < 0.05$ vs. serum-starved MC). The increase in staining did not represent an increase in total MC number, as the number of MC did not increase during the time points chosen in the current study. Figure 7 shows a Western blot analysis for CDK2, and shows that similar to immunostaining, serum-starved MC express low levels of CDK2. In contrast, MC proliferation was associated with a substantial increase in CDK2 protein when stimulated with PDGF (Fig. 7) and bFGF. Figure 8 shows the results of immunoprecipitation studies when mesangial cell protein was immunoprecipitated with an antibody to cyclin A, and a Western blot analysis was performed with an antibody to CDK2. Cyclin A associated with CDK2 during PDGF stimulation at 18 hours, when MC proliferation was significantly increased.

p27 expression is reduced during PDGF and bFGF induced mesangial cell proliferation

Quiescent MC express high levels of p27 protein. Immunostaining revealed that all quiescent MC expressed p27 as shown by the dense intranuclear staining. Figure 9A shows a Western blot analysis for p27 protein. Quiescent MC express p27 and the protein levels did not change at 12 hours of PDGF stimulation, when MC proliferation has not increased. In contrast, there was a decrease in the levels of p27 at 18 hours and 24 hours following PDGF stimulation, which was coincident with the onset of PDGF induced MC proliferation. However, p27 expression was always detectable. Figure 10A shows that the onset of MC proliferation induced by bFGF was also associated with a reduction in levels for p27, but in contrast to the levels during PDGF stimulation, the decrease in p27 levels occurred earlier, and was more pronounced than that observed for PDGF.

We performed immunoprecipitation studies to determine if p27 associated with cyclin A-CDK2 in quiescent and proliferating MC. Figure 11 shows that the CKI p27 is bound to cyclin A-CDK2 in quiescent MC. However, PDGF stimulation causes the dissociation of p27 from this complex, so that no p27 was complexed to cyclin A-CDK2 during MC proliferation at 12 and hours.

TGF-β1 decreases cyclin A expression, and prevents the decrease in p27 levels

As has been shown by others [13, 14], TGF-β1 in the current study reduced but did not completely prevent PDGF and bFGF induced MC proliferation (Figs. 1, 2, 3 and 4). Figures 5 and 6 show that TGF-β1 prevents the maximal increase in cyclin A expression at 24 hours that is induced by PDGF and bFGF respectively. In contrast, TGF-β1 did not affect the levels of CDK2 in PDGF or bFGF stimulated MC (results not shown).

The levels of p27 during TGF-β1 mediated reduction of PDGF and bFGF induced MC proliferation was determined by Western blot analysis, and are shown in Figures 9 and 10, respectively. In MC stimulated with PDGF, TGF-β1 prevented the decline in p27 levels at 18 hours and 24 hours (Fig. 9B) that was observed when MC were stimulated with PDGF alone. In MC stimulated with bFGF, TGF-β1 not only prevented the decline in p27 levels at 18 hours and 24 hours, but appeared to actually increase the levels at 24 hours (Fig. 10B).

Because the association of p27 with cyclin A-CDK2 was decreased by PDGF and bFGF, we performed co-immunoprecipation studies to determine if TGF-β1 prevented this dissociation. Figure 11 shows that in the during the reduction of mesangial cell proliferation by TGF-β1, p27 was complexed with cyclin A-CDK2.

Decreasing the levels of p27 is associated with an increased proliferative response to PDGF and bFGF

We used antisense oligodeoxynucleotides (ODN) to reduce the expression of p27 in rat MC, while mismatch ODN and untransfected MC were used as controls. Using FITC-labeled ODN, we determined that greater than 80% of MC were transfected

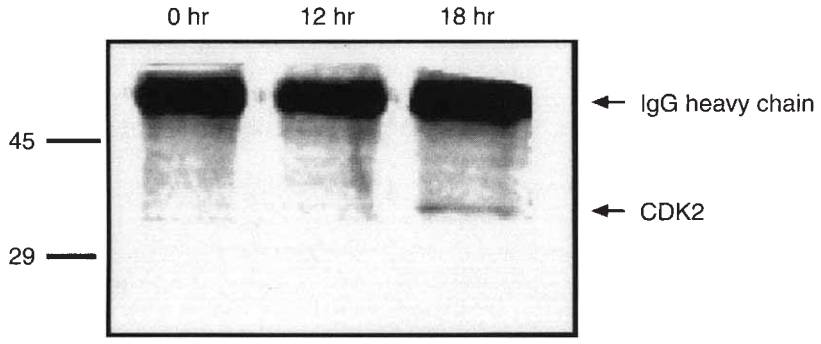

Fig. 8. *Immunoprecipitation of cyclin A.* The protein from serum-starved and PDGF stimulated mesangial cells was immunoprecipitated with an antibody to cyclin A, seperated on a 15% SDS-PAGE, and a Western blot analysis was performed with an antibody to CDK2. Cyclin A complexes with CDK2 at 18 hours of PDGF stimulation (lane 3), which coincides with a significant increase in mesangial cell proliferation. Cyclin A did not complex with CDK2 in serum-starved mesangial cell (lane 1) or following 12 hours of PDGF stimulation (lane 2).

(results not shown). MC cytotoxicity associated with transfection was examined by measuring LDH release at 6 and 24 hours. Transfecting MC with ODN at concentrations greater than 5 nmol caused cell lysis, but this did not occur at a concentration of 1 nmol (results not shown). Cytotoxicity also occurred when MC were transfected with liposomes alone at concentrations greater than 2 μg/ml, but not when transfected with liposome at a concentration of 1 μg/ml.

Based on these results, we selected a concentration of antisense ODN and mismatch ODN of 1 nmol, which was complexed with liposome at a final concentration of 1 μg/ml, and MC were transfected for six hours. Figure 12 shows the levels of p27 protein expression determined by Western blot analysis in MC that were serum-starved and in MC stimulated by mitogens. In MC transfected with mismatch ODN the levels of p27 were identical to non-transfected MC. In contrast, p27 protein expression was markedly reduced in MCs transfected with antisense ODN. The levels of p27 in transfected MC were also determined by Western blot analysis following PDGF and bFGF stimulation. The levels of p27 were reduced in proliferating MC induced by PDGF that had been transfected with mismatch. In contrast, there was a greater reduction in p27 levels in proliferating MC that had been transfected with antisense. Figure 12 also shows that p27 levels were reduced by antisense when TGF-β1 was added to PDGF stimulated cells. To ensure that the levels of other cell-cycle proteins that we had shown to be important in MC proliferation were not altered by transfection, we measured the expression of cyclin A and CDK2. As shown in Figure 13, the levels of cyclin A and CDK2 were not altered by transfecting MC with antisense or mismatch. Furthermore, the levels of cyclin A and CDK2 were not altered in transfected MC exposed to TGF-β1 (results not shown).

The effects of p27 levels on MC proliferation induced by PDGF are shown in Figures 1 and 3. Figure 1 shows that reducing the levels of p27 with antisense ODN resulted in an increased magnitude of MC proliferation as measured by ^{3}H-thymidine incorporation following stimulation with PDGF at 20 hours compared to MC transfected with mismatch ODN (2236 ± 125.5 vs. 1064 ± 46.5 cpm/μg protein, $P < 0.05$) and non-transfected MC (2236 ± 125.5 vs. 1166 ± 100 cpm/μg protein, $P < 0.05$). At 24 hours of PDGF stimulation, MC proliferation remained increased in the antisense transfected MC compared to mismatch transfected MC (2297 ± 300 vs. 1673 ± 26.5 cpm/μg protein, $P < 0.05$) and non-transfected MC (2236 ± 125.5 vs. 1816 ± 81.8 cpm/μg protein, $P < 0.05$). Of interest, in MC transfected with antisense ODN, there was no significant difference in proliferation following PDGF stimulation at 20 hours compared to 24 hours (2297 ± 300 vs. 2236 ± 125.5 cpm/μg protein), whereas in untransfected MC, proliferation was increased at 24 hours compared to 20 hours (1816 ± 81.8 vs. 1166 ± 100 cpm/μg protein,

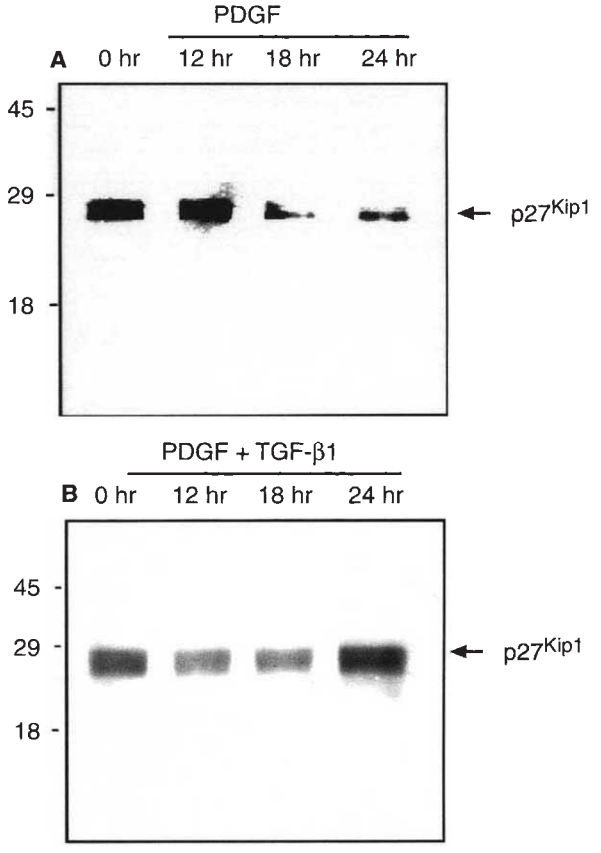

Fig. 9. A. *Western blot analysis for $p27^{Kip1}$ following PDGF stimulation.* Serum-starved mesangial cells (MC) were stimulated with PDGF (10 ng/ml). For Western blot analysis, 20 μg of MC protein was loaded in each lane. Quiescent MC (lane 1) have high levels of $p27^{Kip1}$ (p27) expression, represented by a protein with a molecular weight of 27 kDa. These levels are maintained at 12 hours (lane 2) following PDGF stimulation. There is a decrease in p27 protein levels at 18 hours (lane 3) and 24 hours (lane 4) following PDGF stimulation, and the decrease coincides with the onset of MC proliferation. Protein molecular weight markers (in kDa) is shown in the left margin. **B.** Western blot analysis for $p27^{Kip1}$ during reduction of PDGF induced proliferation by TGF-β1. Serum-starved rat MC were stimulated with PDGF (10 ng/ml) and TGF-β1 (1 ng/ml). In contrast to the decrease $p27^{Kip1}$ (p27) levels in MC stimulated to proliferate with PDGF alone, the levels for p27 do not decrease when TGF-β1 is added to reduce proliferation. Protein molecular weight markers (in kDa) is shown in the left margin.

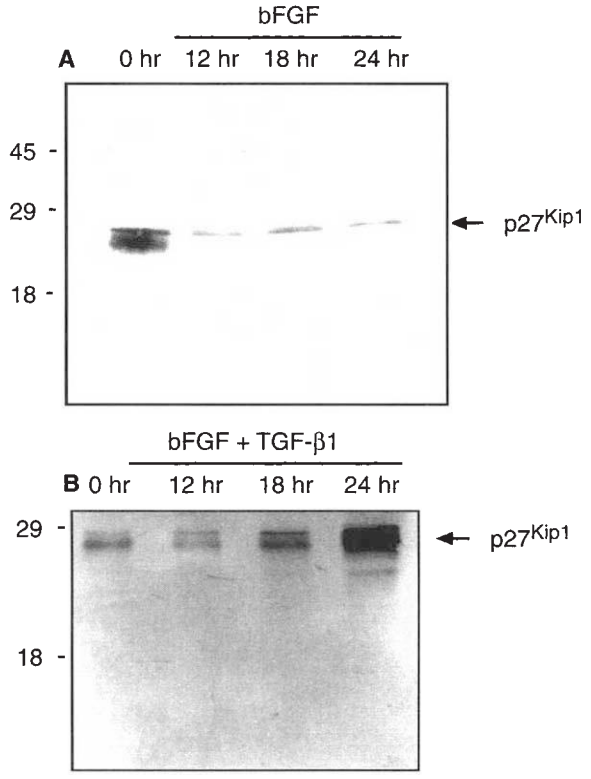

Fig. 10. A. *Western blot analysis for $p27^{Kip1}$ following bFGF stimulation.* Quiescent mesangial cells (MC) were stimulated with bFGF (10 ng/ml). For a Western blot analysis, 20 μg of MC protein was loaded in each lane. Quiescent MC (lane 1) have high levels of $p27^{Kip1}$ (p27) expression, represented by a protein with a molecular weight of 27 kDa. There is a decrease in p27 protein levels at 12 hours (lane 2), 18 hours (lane 3) and 24 hours (lane 4) following bFGF stimulation, and the decrease coincides with the onset of MC proliferation. Protein molecular weight markers (in kDa) is shown in the left margin. **B.** Western blot analysis for $p27^{Kip1}$ during reduction of bFGF induced proliferation by TGF-β1. Quiescent rat MC were stimulated with bFGF (10 ng/ml) and TGF-β1 (1 ng/ml). In contrast to the decrease $p27^{Kip1}$ (p27) levels in MC stimulated to proliferate with bFGF alone, the levels for p27 do not decrease when TGF-β1 is added to reduce proliferation. Protein molecular weight markers (in kDa) is shown in the left margin.

$P < 0.05$). This indicates that the onset of maximal MC proliferation following PDGF stimulation occurred earlier when the p27 levels were lowered with antisense ODN. Similar results were obtained using BrdU staining. As shown in Figure 3, in MC stimulated with PDGF, there was a significant increase in BrdU staining in MC transfected with antisense compared to both non-transfected MC (56 ± 1.0% vs. 40 ± 1.5%, $P < 0.05$) and MC transfected with mismatch (56 ± 1.0% vs. 41 ± 1.9%, $P < 0.05$). Although there was a trend towards increased MC proliferation at 12 and 16 hours of PDGF stimulation, there was no significant difference in proliferation between the antisense transfected MC compared to control (results not shown).

The effects of p27 levels on MC proliferation induced by bFGF are shown in Figures 2 and 4, respectively. The magnitude of the MC proliferative response measured by ^{3}H-thymidine incorporation into DNA (Fig. 2) was greater at 20 hours in MC transfected with antisense compared to non-transfected MC (2747 ± 275 vs. 1462 ± 95 cpm/μg protein, $P < 0.0001$) and mismatch transfected MC (2747 ± 275 vs. 1522 ± 95 cpm/μg protein, $P < 0.0001$). In contrast to PDGF, there was no significant difference in MC proliferation after 24 hours of bFGF stimulation in MC transfected with antisense ODN compared with controls. Similar to the results seen with PDGF, reducing the levels of p27 was not associated with increased proliferation at 12 hours or 16 hours of bFGF stimulation (results not shown). However, the onset of maximal MC proliferation following bFGF stimulation was earlier when the levels of p27 were reduced by antisense compared to controls. Figure 4 shows the results of BrdU staining in MC stimulated with bFGF. When MC were stimulated with bFGF there was a marked increase in BrdU staining in MC with following transfection with antisense ODN compared to non-transfected MC (74 ± 2.4% vs. 42 ± 1.9%, $P < 0.05$) or MC transfected with mismatch (74 ± 2.4% vs. 40 ± 2.8%, $P < 0.05$).

The proliferative effects of reducing p27 levels in MC depended on the presence of mitogens. Our results show that there was no difference in ^{3}H-thymidine incorporation (Fig. 1) or BrdU staining (Fig. 3) between the MC transfected with antisense compared to mismatch ODN and untransfected MC controls when serum-free media was used. However, if MC were transfected with ODN while plated in 15% FCS (as opposed to serum-free media), there was an increased proliferation in MC transfected with antisense ODN compared to control MC transfected with MSM OSN or untransfected MC ($P < 0.05$).

We also studied the role of p27 on the effects of TGF-β1 on PDGF and bFGF stimulated MC proliferation using ^{3}H-thymidine and BrdU incorporation, and the results are shown in Figures 1 to 4. In MC transfected with antisense or mismatch and in non-transfected MC, the addition of TGF-β1 reduced but did not prevent MC proliferation induced by PDGF or bFGF ($P < 0.05$ PDGF alone, $P < 0.05$ vs. bFGF alone). Furthermore, there was no significant difference in ^{3}H-thymidine or BrdU incorporation in transfected MC compared to non-transfected MC.

Discussion

We examined cell-cycle protein response in the glomerular MC to various cytokines that have been shown to be important in mediating MC proliferation *in vitro*. Although much is known about cell-cycle proteins in non-renal cells, it is clear that the expression of cell-cycle proteins during mitogenesis can vary in individual cell types, and also may vary within a cell type depending on the mitogen. We therefore began our studies by determining the expression of various cell cycle proteins in the glomerular MC in response to two mitogens, PDGF and bFGF. These mitogens were chosen as there is substantial evidence that both play a major role in the proliferative response of the MC both *in vitro* and *in vivo* in the development of disease [2, 3].

Expression of cell-cycle proteins in response to mitogens (PDGF and bFGF)

As previously shown, both PDGF [4, 5, 12] and bFGF [6] induced substantial MC proliferation peaking at 24 hours. In the current study we serially examined the expression cell-cycle proteins during the 24 hours of mitogen stimulation to determine the expression of G1-S cyclins. Cyclin A increased at 12 hours, consistent with later G1, and similar to that observed in other cell types [18]. However, the increase in CDK2 expression was a bit unexpected when compared to non-renal cells, but consistent with

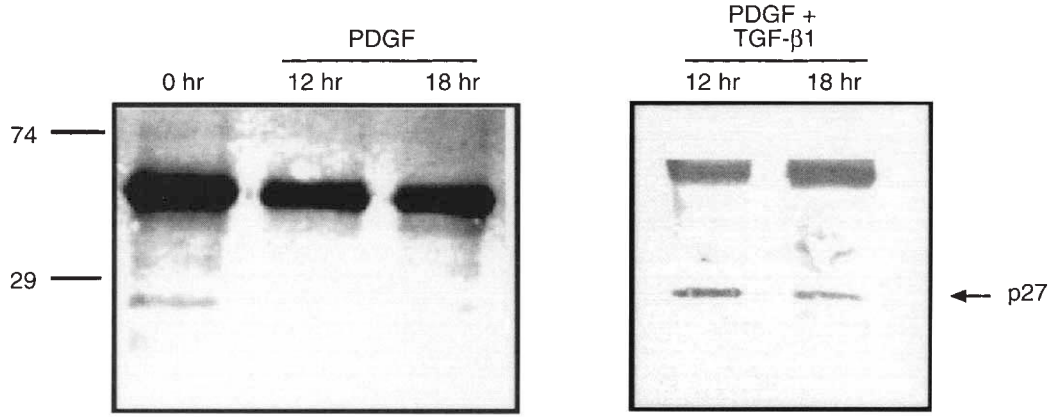

Fig. 11. *Co-immunoprecipitation of p27 and cyclin A-CDK2.* Mesangial cell protein was immunoprecipitated with an antibody to cyclin A, separated on a 15% SDS-PAGE, and a Western blot analysis was performed with an antibody to p27. In serum-starved mesangial cells (lane 1), p27 was complexed with cyclin A-CDK2, and the association was lost in PDGF stimulated mesangial cells at 12 hours (lane 2) and 18 hours (lane 3). Molecular weight markers (kDa) are shown in the left margin. p27 was complexed with cyclin A CDK2 in TGFβ-1 stimulated mesangial cells.

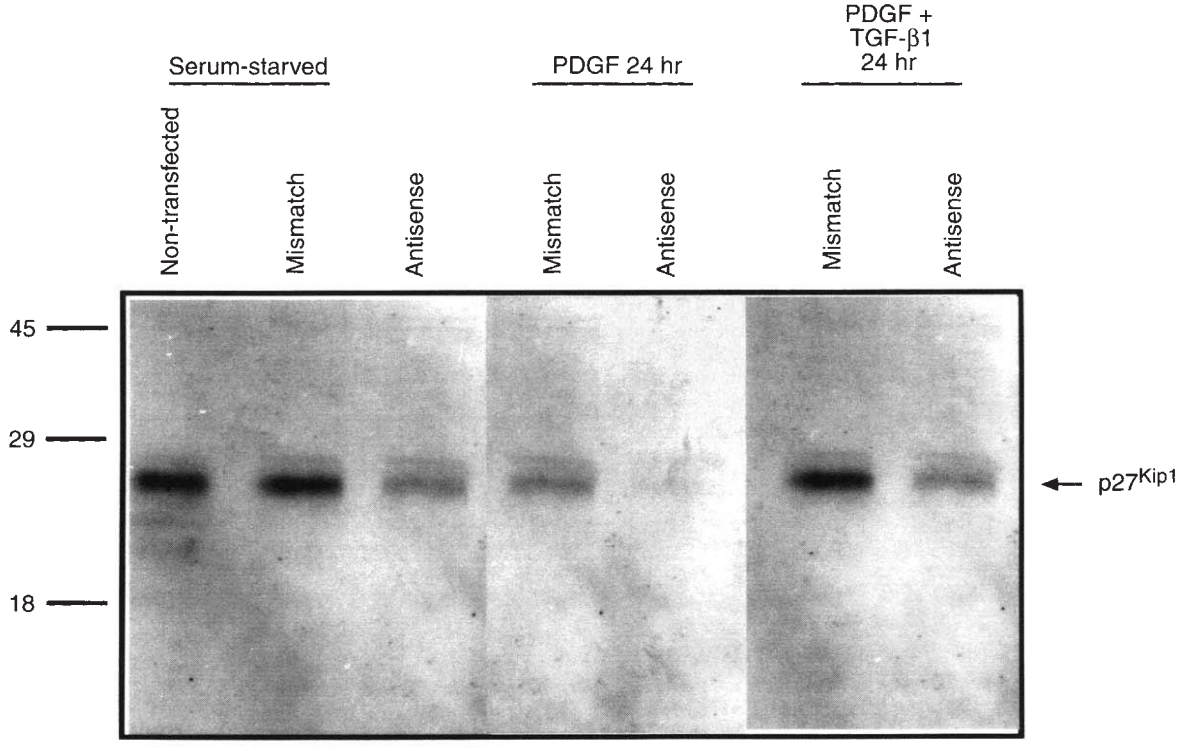

Fig. 12. *Western blot analysis for $p27^{Kip1}$ (p27) in mesangial cells transfected with specific oligodeoxynucleotides (ODN).* The level of p27 was determined by Western blot analysis in non-transfected mesangial cells (MC) and MC transfected with mismatch oligodeoxynucleotides to p27 and with antisense oligodeoxynucleotides to p27. The levels for p27 were high in serum-starved non-transfected MC (lane 1) and in MC transfected with mismatch (lane 2). In contrast, the levels for p27 were reduced in MC transfected with antisense p27 (lane 3). Stimulating MC to proliferate with PDGF (10 ng/ml) resulted in a decrease in p27 levels in mismatch transfected MC (lane 4), but the levels were markedly decreased in MC transfected with antisense (lane 5). TGF-β1 did not increase the levels of p27 in mismatch transfected (lane 6) or antisense transfected (lane 7) mesangial cells.

our recent *in vivo* finding in the Thy1 model of experimental glomerulonephritis [29]. The levels of p27 were reduced by PDGF and bFGF. Similar results for PDGF has been shown by Coats [27]. Of interest was the finding that the more potent MC mitogen, bFGF, was associated with a more substantial and earlier reduction in p27 levels compared to PDGF. This observation suggested that the more pronounced reduction in p27 levels by bFGF may account for increased MC proliferation compared to PDGF stimulation. Furthermore, MC proliferation was associated with a dissociation of this cyclin kinase inhibitor from the cyclin A—CDK2 complex.

The levels of p27 in proliferating cells may be cell type specific, and also may be determined by the mitogenic stimulus. For example, Mv1Lu lung epithelial cells and human keratinocytes

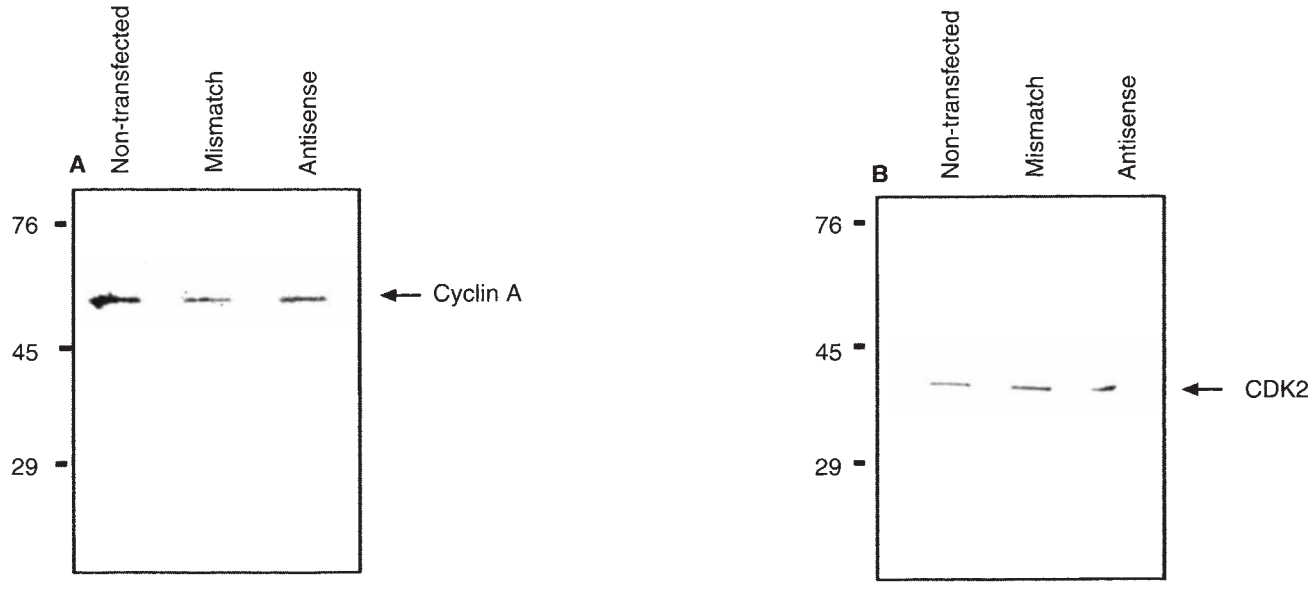

Fig. 13. *Western blot analysis for cyclin A and CDK2 in transfected mesangial cells (MC).* The levels for cyclin A (**A**) and cyclin dependent kinase 2 (CDK2) (**B**) are unchanged in proliferating MC stimulated by PDGF when transfected with mismatch oligonucleotides (lane 2) or with antisense oligonucleotides (lane 3) compared to non-transfected MC (lane 1).

retain a high level of p27 in their proliferative state, whereas the levels of p27 in T cells, macrophages [30] and fibroblasts [31] decline when stimulated to enter the cell cycle. However, in contrast to the undetectable levels for p27 in fibroblasts, p27 levels were always detected in proliferating MC. In human T cells, p27 maintains quiescence, and IL-2 induced proliferation in this cell type is associated with a decrease in p27 expression, which is prevented by rapamycin [31]. More recently Coats and colleagues showed that IGF-1 and EGF stimulation of Balb/c-3T3 cells was not associated with a decrease p27 in expression [27]. One possible explanation why there is only a partial decline in p27 levels with PDGF and bFGF induced MC proliferation in the current study may reflect the fact that less than 40% of MC proliferate in response to the mitogens used in this study, whereas a higher number proliferate in other cell types. A second possibility may be that different mitogens have a varied effect on p27. The mechanism whereby p27 protein levels are reduced in MC are unknown, but recent evidence suggests that there is increased degradation of this protein during proliferation through the ubiquitin pathway [32]. However, it is not known if PDGF or bFGF directly increases p27 degradation.

Effect of TGF-β1 on reducing MC proliferation

TGF-β1 is an important cytokine thought to play a major role in glomerular disease due to its ability to stimulate matrix [33, 34]. However, another important function of TGF-β1 is its ability to reduce proliferation of MC that have been stimulated with mitogens such as PDGF and bFGF [3, 12]. Similar to that shown by Jaffer et al [12], in the current study we show that rat MC proliferation induced by PDGF and bFGF is reduced, but not completely suppressed by TGF-β1. This effect was associated with a reduction in cyclin A, but not CDK2 expression. The effect of TGF-β1 on cyclin A has been shown in other cell types such as in Mv1Lu cells [35], although more recent studies in this cell type suggest that the decline in CDK2 activity by TGF-β1 is more likely due to a cyclin kinase inhibitor [24]. These authors showed that TGF-β1 increased the CKI, p15, which displaced p27 from cyclin D-CDK4 complexes, allowing p27 to inhibit cyclin E-CDK2 complexes. The effects of TGF-β1 on different cyclins varies, and TGF-β1 does not affect the levels of cyclin D1 or cyclin E [36, 37]. In the current study we showed that during inhibition of MC proliferation, TGF-β1 prevented the decrease in p27 levels induced by PDGF and bFGF. Moreover, TGF-β1 increased p27 levels at 24 hours, when MC proliferation is maximal in the absence of TGF-β1. In addition to preventing a decrease in p27 levels in mesangial cells following mitogen stimulation, we showed that TGF-β1 also prevented the dissociation of p27 from cyclin A-CDK2 complexes during the reduction of proliferation. Because these results suggested to us that TGF-β1 may reduce mesangial cell proliferation through p27, functional studies were undertaken to address this hypothesis (see below).

Functional Studies of p27 in MC

The earlier experiments in the current study suggested that p27 may have a critical role in both PDGF and bFGF induced proliferation (where p27 levels fall and dissociate from cyclin A—CDK2), and for TGF-β1 reduction in proliferation (where p27 levels maintained). However, while these results are important in understanding the expression of specific cell-cycle proteins in MC, these studies were descriptive. Therefore, we undertook functional studies with antisense to p27 to determine the role of this cyclin kinase inhibitor in MC proliferation. Antisense reagents have been successfully used to inhibit the synthesis of a wide range of proteins [38, 39], including those involved in cell-cycle progression such as CDC2 [40], *myc* [41] and *myb* [42]. Antisense inhibition of cyclin B synthesis was one of the first demonstrations that cyclin synthesis was necessary for entry into mitosis [43]. More recently, Coats et al used the same antisense, mismatch and liposomes used in this study to show that p27 is required for restriction point control in fibroblasts [27]. In the

current study we used mismatch oligodeoxynucleotides, which have a similar sequence to the antisense, but the bases are "scrambled." Because of potential cytotoxicity induced by transfection, we ensured that the experimental conditions used did not cause toxicity.

We show three major findings. First, in the absence of p27, PDGF and bFGF proliferation in rat MC is of greater magnitude, suggesting that p27 plays a critical role in the response to these mitogens. Furthermore, the onset of maximal MC proliferation is increased when the levels of p27 are reduced with antisense compared to control. Second, lack of p27 by itself is not sufficient to allow MC proliferation. Hence, serum starvation alone showed no increased proliferation when p27 was eliminated. These results suggest that proliferation requires both the loss of p27 and the presence of a mitogen. Third, despite maintaining p27 levels, TGF-β1 does not require p27 to reduce the proliferative effect of PDGF and bFGF on rat MC. However, effects of TGF-β1 on other cell-cycle proteins, such as p15 and $p21^{CIP1}$, should be undertaken, as it has been shown by Morrissey et al that TGF-β1 increases $p21^{CIP1}$ in the renal cortex *in vivo* [44].

In conclusion, we identified some of the cell-cycle regulatory proteins that are activated in MC during proliferation. We have shown a significant role for p27 in the proliferative response to PDGF and bFGF. However, the maintainance of p27 by TGF-β1 is not critical by itself to block proliferation. These latter findings are new to the cell-cycle in general. Further studies are needed to determine the role of other cyclin kinase inhibitors in the proliferative response.

Acknowledgments

Support for this study was provided by Public Health Service grants (DK 34198, DK43422, DK 51096, P50-47659). S.J.S. was also supported by a grant-in-aid from the American Heart Association, Washington Affiliate (97354).

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Appendix

Abbreviations are: MC, mesangial cell; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; TGF-β1, transforming growth factor-β1; ODN, oligodeoxynucleotide; MSM, mismatch; p27, $p27^{Kip1}$.

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